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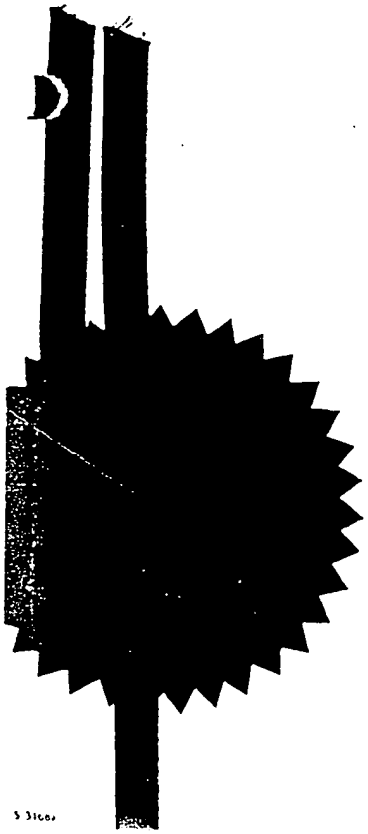

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REQUEST FOR GRANT OF A PATENT

THE GRANT OF A PATENT IS REQUESTED BY THE UNDERSIGNED ON THE BASIS OF THE PRESENT APPLICATION

I Applicant's or Agent's Reference *(Please insert if available)* HGH/AMP

II Title of Invention Antibodies

III Applicant or Applicants *(See note 2)*

Name (First or only applicant) MEDICAL RESEARCH COUNCIL

Country United Kingdom State United Kingdom ADP Code No. 5960070

Address 20 Park Crescent, London, W1N 4AL.

Name (of second applicant, if more than one)

Country State

Address

IV Inventor (see note 3) [redacted]

(b) A statement on Patents Form No 7/77 is/will be furnished

V Name of Agent (if any) *(See note 4)* CARPMAELS & RANSFORD ADP CODE NO 53001

VI Address for Service (See note 5) 43, BLOOMSBURY SQUARE
LONDON, WC1A 2RA

VII Declaration of Priority (See note 6)

Country	Filing date	File number

THE PATENT OFFICE
10 OCT 1990

VIII The Application claims an earlier date under Section 8(3), 12(6), 15(4), or 37(4) (See note 7)

Earlier application or patent number ... and filing date

IX Check List (*To be filled in by applicant or agent*)

A This application includes the following number of sheet(s):

1	Request	1	Sheet(s)	1	Priority document
2	Description	21	Sheet(s)	2	Translation of priority document
3	Claim(s)	–	Sheet(s)	3	Request for Search
4	Drawing(s)	6	Sheet(s)	4	Statement of Inventorship and Right to Grant
5	Abstract	–	Sheet(s)		

B The application is not accompanied by:

X It is suggested that Figure No 1 of the drawings (if any) should accompany the abstract when published.

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At present, we can genetically engineer monoclonal antibodies and endow them with new properties. In the future, gene technology may provide the means for making antigen-binding fragments by exploiting repertoires of V-genes derived from immunised animals and expressed in bacteria. How readily can this approach be extended to production of 'in vitro' repertoires of V-genes, and obviate the immunisation of animals ?

In 1975 a method was described for making cell lines which secrete a single species of antibody (monoclonal antibody or mAb) with the desired specificity to antigen[1]. Antibody-producing cells from a mouse immunised with sheep-red blood cells were immortalised by fusion with a myeloma and screened for those secreting antibodies to sheep red blood cells. The technique - "hybridoma technology" - proved general and a wide range of monoclonal antibodies have been made with binding activities to protein, carbohydrate, nucleic acids and hapten antigens, and even catalytic activities[2,3], leading to many practical applications of mAbs in research and human health-care[4-7] and patent disputes[8]. The technology has improved over the years, particularly by preselecting antigen binding B-cells[9] and by screening with antigen coated filters[10].

A further dimension to hybridoma technology was introduced by somatic cell genetics, allowing antibody mutants to be selected[11,12], their functional properties to be changed by switching heavy chain constant regions[13] and antibodies to be made with dual specificity[14]. Gene technology revolutionised this potential as antibody genes could now be altered to order. New vistas appeared, reviving the forgotten excitement of the old discipline of immunochemistry of antibodies. Initially antibody genes were taken from hybridomas, cloned into plasmid vectors and expressed as complete antibodies in mammalian cells[15,16]. Later antibody fragments were expressed from bacteria[17-20]. The ready manipulation of the genes by cutting and pasting of restriction fragments, or by site directed mutagenesis, has allowed the construction of new antibody reagents and fine mapping of antibody structure-function relationships.

More recently a new approach to clone antibody genes was proposed with the potential to bypass hybridomas[21,22]. The genes are cloned directly from lymphocytes of immunised animals, expressed in bacteria and the antibody products screened for binding to antigen[23,24]. Like hybridoma technology, the process relies on animal immunisation to give rise to a large number of antigen-

specific cells. In the animal, antibodies of low affinity are first produced by antigen-induced proliferation of cells, and then higher affinity variants are created by point mutation and selection. Hybridoma technology can immortalise these cells; gene technology can immortalise their genes. In both cases animals "invent" the new molecules.

Looking ahead, can we even bypass animals and make new antibodies in vitro[25]? Two current strategies for making new antibodies recapitulate the great immunological controversy of the 1950's - instruction versus selection[26]. The modern instructionist uses computer graphic techniques to build specific antigen-binding sites. The selectionist attempts to make repertoires of antibody genes and select those with antigen-binding activity, so mimicking nature.

Starting with hybridomas

The antibody (IgG) is a Y-shaped molecule, in which the tips of the arms bind to antigen and the stem is responsible for triggering effector functions such as complement or cell-mediated killing. The domain structure of the molecule makes it particularly accessible to protein engineering, as functional domains carrying antigen-binding activities (Fv, Fab fragments) or effector functions (Fc fragments) are readily excised as fragments, or swapped between antibodies[27,28] (see Fig. 1 and legend). Furthermore, the rigid β–sheet framework structure of the V-domains, surmounted with antigen-binding loops allows the transplanting of binding sites from one antibody to another[29]. These structural features have spawned a range of designer antibodies, from complete antibodies equipped with natural effector functions to antigen-binding fragments with attached radioisotopes or toxins (Fig. 2).

Antibodies kill by triggering complement or cell-mediated lysis. C1q, the first component of complement, binds to clusters of antibody, such as would form on a surface studded with antigenic epitopes, and triggers the complement cascade. Likewise specialised effector cells, such as phagocytes or killer cells, can bind to antibody clusters through cell receptors. This triggers phagocytosis or antibody-dependent cell-mediated cytolysis (ADCC). The potential of an antibody in lysis is determined mainly by the class of constant domains (isotype). This has been dissected by making chimaeric antibodies, in which the variable domains of a rodent antibody were attached to the constant domains of human γ isotypes. The human γ1 isotype emerges as highly effective in both

complement and cell-mediated killing, and therefore the most suitable for therapy[30,31]. Conversely, the inactive human γ4 isotype may be more suitable for imaging and blocking[32]. Analysis of the roles of individual amino acid residues should permit the engineering of variants of a single antibody isotype with differing effector mechanisms. Thus the binding sites for the high affinity receptor (FcRI) include the lower hinge region of the antibody[33], and the "core" binding site for C1q is a strand of β-sheet in the CH2 domain[34] (Fig.1).

Although some rodent antibodies (particularly the mouse γ2a and rat γ2b isotypes), can trigger human effector mechanisms, they are immunogenic in human therapy. In view of the difficulties of making human monoclonal antibodies directly (see later), rodent antibodies have been "humanised" by linking rodent variable regions and human constant regions[28] (Chimeric antibodies Fig. 2). This reduces the immunogenicity of the antibody as shown in clinical trials[35]. However, residual immunogenicity is retained (at least in part) by virtue of the foreign V-region framework[36].

A more complete way of humanising rodent antibodies includes the replacement of the V-region framework ("reshaped" antibodies, Fig. 2). It relies on the architecture of V-domains as a framework of β-sheets topped with antigen-binding loops (see ref. 37 and Fig. 1). By grafting the loops, the antigen binding site can be transferred from rodent to human antibody[29,31,38,39]. The technique was used to humanise a rat therapeutic antibody directed against mature human leucocytes[31] which proved clinically effective in destroying a large mass of tumour in two patients[40]. However, reshaping requires that the different framework regions are structurally conserved, both in the orientations of the two β-sheets of each domain and in the packing of VH and VL domains together; that the hypervariable loops make the majority of contacts with antigen, and that the loops are supported in a similar way by the underlying β-sheet framework. Although these are likely to be true for some antibodies, the restitution of key contacts between loops and framework has proved necessary in others. Simple molecular modelling can help identify contacts and design small changes to optimise affinities[31,41].

Although natural effector functions are powerful, antibodies can also be engineered to recruit other effector functions. For example, antibodies with dual specificity were made by fusion of hybridomas of two different specificities (hybrid hybridomas)[14,42]. Here the two halves of a single antibody molecule

are from each of the two parental antibodies, and therefore carries two different antigen binding sites. Such bispecific antibodies can bind both to a cell-target (tumour) and to a toxin or a cytotoxic T-cell[43,44]. Novel effector functions can also be recruited by gene fusion of antigen binding sites (as Fv or Fab fragments) to toxins[45] and enzymes[29], allowing the targeting of agents such as tissue plasminogen activator to blood clots, where it can locally activate plasminogen[46]. Fusion of antibodies with enzymes may prove invaluable for activation of prodrugs[7,47]. Conversely antibody Fc fragments can equip other proteins with antibody effector functions. For example, CD4 linked to Fc fragments binds to viral gp120 on the surface of HIV-infected cells, and kills the cells via ADCC (ref. 48) (CD4 immunoadhesin, Fig. 2).

Complete antibodies (Mr = 150,000) are large molecules but much smaller fragments can be prepared that retain antigen binding activity. Small fragments (Mr 12,000 - 50,000) are particularly attractive for *in vivo* therapy as they penetrate tissue boundaries more effectively[49]; fragments are also cleared faster from the serum and tissues[50] and aid the clearance of toxic drugs, such as digoxin, from the circulation[51]. Small fragments also have advantages for high resolution X-ray crystallographic studies of antigen-binding sites. The size of antibodies and flexibility of the hinge connecting Fab arms and Fc domain have prompted crystallographers to turn to Fab fragments[52] and now Fv fragments[53] (Fig. 2). In future we expect that fragments will be used extensively as they are readily expressed in an active form from genes introduced into mammalian[27,54] or bacterial cells[19,20,23,55]. However antibody fragments may require further engineering to eliminate undesirable properties.

For example, Fv fragments are non-covalently associated heterodimers of heavy and light chain variable domains which may thus dissociate. Although some Fv fragments are less prone to dissociation than others[56], stable Fv fragments can be engineered either by linking the domains with a hydrophilic and flexible peptide[57,58] to create single chain Fv fragments (scFv, Fig. 2), or by introducing disulphide bonds between the domains[56]. Single heavy chain variable (VH) domains (dAb, Fig. 2) with antigen binding activities[23] are likely to require more extensive engineering. The domains have an exposed hydrophobic surface (where they normally interact with light chain), rendering them "sticky". If their properties can be improved, for example by introducing hydrophilic residues to the interface, these single domains may prove the forerunners of a new generation of small recognition molecules.

Bypassing hybridomas

Immunisation is essential to derive hybridomas secreting high affinity antibodies. In the animal these antibodies are produced following a maturation of the immune response. This is a two stage process[59,60]. The first stage is fast but leads to the production of antibodies of low affinity (10^5 - 10^7 M^{-1}). It involves a proliferation of cells drawn from the ***available*** repertoire. Potentially this repertoire is huge (>10^{10}) (Fig. 3 and legend) and arises from the genetic recombination of five families of gene fragments, namely VH, VL, DH, JH and JL. At any given time, only a fraction of the ***potential*** repertoire of a mouse is available through the limited number of clones (10^7-10^8) expressing antibodies.

The second stage is designed for the production of high-affinity antibodies, starting with the genes utilised in the first stage. Its main tool is the hypermutation of these genes followed by selection of those cells which produce antibodies of increased affinity. It is a Darwinian process, involving ***variation*** through a process of point mutations and ***selection***, which is driven by competition for antigen as the requirement for cell survival. It is ***inheritable***, but only at the somatic level among the lymphoid cells of the individual. The resulting memory cells seem to be able to undergo new rounds of hypermutation and selection following antigenic challenge[61].

The rate of mutation approaches 10^{-3}, or 3 x 10^{-4} /base pair/cell division in a more recent estimate[60,61,62]. Mutations are localised to the segment coding for the variable portion of the antibody genes. The potential diversity generated by hypermutation is astronomical. Even if only the hypervariable region (~30 residues) of a single antibody could each mutate into 10 out of the 20 amino acids, this would produce about 10^{30} variants for each chain. *So the problem at this stage is not the generation of diversity, but the continuous selection of improvements against a background of degeneracy*[63]. As immunisation proceeds, additional high affinity antibodies gradually emerge with V-D-J combinations which are rarely found in the primary repertoire (repertoire shift)[64].

In "classical" antibody engineering, hybridomas of known specificity have provided the raw material for cloning the rearranged antibody VH and VL

genes. However, using "universal" primers and the polymerase chain reaction (PCR) it is possible to rescue V-genes and by building restriction sites into these primers the amplified DNA can be cloned direcly for expression[21]. This has now been acheived with hybridomas[21,22,65] and splenocytes[23,66] expression in mammalian cells[21] or bacteria[23,24].

The cloning of V-genes directly for expression, offers new routes for the derivation of monoclonal antibody-producing cell lines (Fig. 5). At its simplest, V-genes could be rescued from hybridomas[21], unstable hybridoma fusions (e.g. mouse-human hybridomas), single hybridoma cells[67,68] or even single B-lymphocytes. Hybridomas have advantages as fusion enriches for antigen-stimulated cells while rescue from single B-lymphocytes by-passes cell fusion, allowing access to terminally differentiated B-cells which are rich in mRNA but do not fuse. Single hybridomas or B-cells might also be isolated by binding to antigen, immobilised on solid supports (for example on plates or magnetic beads), by fluorescent activated cell sorting (FACS) or by rosetting with antigen-coated red cells. The single set of V-genes from individual cells could then be co-expressed in bacteria. However each cell must be processed separately.

Cloning from heterogeneous cell populations has the advantage that all the cells can be processed together. For example, VH and VL genes, taken from total unfractionated cells from an immunised mouse have been combined at random and Fab fragments expressed from lambda phage screened for antigen-binding activities[24]. The disadvantage is that the original VH and VL pairing, selected for high affinity by immunisation, is lost. A combinatorial library with only 1000 different VH and 1000 different VL gene elements equally represented, would necessitate the screening of between 10^6 and 5×10^6 clones to recover most of the original pairings. Thus the chances of recovering original pairs of V-genes from a large random combinatorial library from an immunized mouse are low, and remote for the highest affinity antibodies of hyperimmune animals. More likely, pairs of VH and VL domains will be found that are capable of binding antigen[69,70] but perhaps at the expense of the affinity or specificity. The reported isolation[24] of an anti-hapten Fab fragment with a good affinity using such an approach leads to the question as to whether the affinity is as good as could have been recovered from the original VH:VL pair? Indeed, the issue is not just to make binding activities. If the aim of this technology is to

bypass hybridomas, it will need to make the high affinity antibodies required for diagnosis and therapy, difficult to derive even with hybridomas.

To improve the chances of recovering original pairs, the complexity of the combinatorial libraries could be reduced by using small populations of antigen-selected B-lymphocytes (Fig. 5). However all combinatorial approaches will rely heavily on powerful screening methods. Here the use of membrane filters for screening large numbers of clones is promising. A variety of formats have been deployed, for example capture of antibodies on filters coated with antigen, and detection with anti-globulin reagents[10]. Alternatively antibodies or Fab fragments have been immobilised directly on membrane filters[24] or indirectly through antiglobulin reagents (unpublished from this laboratory) and probed with labelled antigen.

Bypassing animals.

Looking ahead, it may become possible to build antibodies from first principles, taking advantage of the structural framework on which the antigen binding loops fold. In general these loops have a limited number of conformations which generate an endless variety of binding sites by alternative combinations and by diverse side chains[71,72,64]. Recent successes in modelling antigen binding sites[72] augurs well for de novo design, but the computer graphic docking of protein antigen to such predicted structures adds a further tier of uncertainty[41]. In any case a high resolution structure of the antigen is needed. However the approach might become attractive for making catalytic antibodies, particularly for small substrates. Here side chains or binding sites for prosthetic groups[73] might be introduced, not only to bind selectively to the transition state of the substrate, but also to participate directly in bond making and breaking. The only question is whether the antibody architecture, specialised for binding, is the best starting point for building catalysts. Genuine enzyme architectures, such as the TIM barrel, might be more suitable. Like antibodies TIM enzymes also have a framework structure (a barrel of β–strands and α-helices) and loops to bind substrate. Many enzymes with a diversity of catalytic properties are based on this architecture[74] and the loops might be manipulated independently of the frameworks[75] for design of new catalytic and binding properties.

Instead of the "design and build" approach, could we build an artificial selection system, for example harnessing bacteria or phage, to select for antigen binding

activities? Here no structural information about the antigen is needed. However, we see no future in trying to select high affinity binding activities in a single step. The strategy of the immune system, in which low affinity evolves to high affinity seems more realistic[25]. Can we imitate this strategy and indeed improve on it?

Our first task is to prepare a naive repertoire of antibody genes (Fig. 6). At its simplest we could use the PCR and universal primers to reproduce *in vitro* the repertoire of rearranged V-genes expressed by naive animals. However naive animals are not naive and the available repertoire is limited in size and shaped, for example by tolerance mechanisms to self-epitopes. In principle we could adopt the version of the strategy used by mammals and assemble a much larger repertoire by random combination between restriction fragments encoding the germline V, D and J elements. To match the potential repertoire of the animal, we would also have to reproduce the junctional diversity created by recombination. Large repertoires might also be made by trying to imitate the process of gene conversion adopted by birds[76].

However such "natural" repertoires are not ideal. The sequences of germline V-genes and the multiplicity of highly related V-genes in the genome are presumably themselves the result of chance and evolutionary pressures, for example towards pathogens and against self-components. Such repertoires are both biased and highly redundant. More efficient repertoires might be constructed by using V-genes from a variety of animal sources, excluding highly related V-genes and even designing entirely new V-genes or D-segments. For example the structures of individual antibody loops are often very similar in their overall fold[71,72], and the loops could be fleshed with diverse side chains.

The next stage is to express the library and screen for antigen-binding activities by random combination of VH and VL domains. Ideally the library should be "complete", containing antibodies of a minimum binding affinity for any conceivable epitope. The tighter the binding of a primary antibody, the larger the library required. For example, it has been estimated that a primary repertoire of 10^7 different antibodies is likely to recognise over 99% of epitopes with an affinity constant of 10^5 M^{-1} or better, and rarely contributes high affinity antibodies to an epitope taken at random (> 10^9 M^{-1}) (ref. 77). If millions of VH and VL combinations from an artificial library could be screened, for

example on membrane filters with a cut-off affinity of at least 10^5 M^{-1}, the library would be as complete as the primary repertoire of a single mouse (10^7 antibody species). In the future, the screening of large libraries may well be replaced by methods of selection. If these screening and selection techniques worked as well as those in the animal, we could imitate the primary response and generally obtain low affinity antibodies.

The enhancement of affinity *in vivo* can be contributed by a single point mutation, or several mutations[64]. Hypermutation of the genes corresponding to antibodies of low affinity should be easy to imitate *in vitro*. Point mutations could be introduced into the V-genes by a wealth of techniques, for example, by using error-prone polymerases, PCR amplification through a large number of cycles, biased ratios of nucleotide triphosphates or "spiked" oligonucleotide primers. Multiple mutations could be targetted throughout the body of the gene simultaneously, or to each of the hypervariable loops[79].

The screening or selection of the mutants with improved affinity is likely to be more difficult, as it needs to discriminate between mutants differing slightly in affinity. Such discrimination is possible for hybridoma clones in agarose[10], by binding secreted antibody on coated membranes with different antigen density. The differential binding to antigen-coated membranes might also be achieved by competition with low affinity antibody. However the system we are proposing is quite primitive compared with the animal. Ideally we would like to hypermutate specific segments of DNA within cells rather than in isolated DNA, and at the same time express the products on the cell surface, and select in a Darwinian fashion variants of steadily increasing affinity. Animals are superb at this job, and it will not be easy to compete with their efficiency. We may learn to imitate the animal strategy, but in the meantime for the production of high affinity antibodies we will normally do better by stealing hypermutated V-genes from immunised animals.

Human antibodies: today and tommorrow

Making human mAbs has posed difficulties for hybridoma technology. For example, the use of the mouse myeloma as a fusion partner for human cells leads to preferential loss of human chromosomes, and intolerable instability of

the hybrids. Hybridomas are derived from spleen or lymph nodes, while the primary source from humans, the peripheral blood lymphocytes, contain few blast cells actively involved in the immune response. As an alternative to fusion, EBV immortalisation of human cells does not lead to preferential immortalisation of blasts engaged in antibody responses, and also leads to lines which are low antibody-producers and rather unstable[80]. Furthermore, humans can rarely be hyperimmunised to order, especially with noxious chemicals, pathogenic viruses or cancer cells. In any case the isolation of human antibodies to human cell surface antigens would have to overcome tolerance mechanisms which eliminate lymphocytes with self-reactivity. Recently, human lymphocytes have been used to populate severe combined immunodeficient (SCID) mice and these animals can be immunised[81,82] to make human antibodies.

Gene technology offers alternatives. The "humanising" of rodent mAbs is currently the most practical approach. It allows access to a vast pool of rodent antibodies with good affinities and specificities. This is a major advantage, particularly when dealing with antibodies for tumour therapy, or for the *in vivo* manipulation of the human immune system. Thousands of antibodies have already been made against human cell surface antigens, and particularly against human leucocytes[4]. Reshaping these antibodies, by transplanting only the antigen-binding loops to human antibodies[29,31], yields humanised antibodies which may have similar immunogenicity to truly human antibodies. In future several other approaches may become available through gene technology as discussed in earlier sections. Furthermore, transgenic mice have been made which carry Ig heavy V, D, J and human constant regions[83] and this should allow human antibodies to be produced directly from hyper-immunised mice. It remains to be seen whether the size of the repertoires, limited by the amount of new DNA that can be carried by the transgenic animal, will constitute a drawback.

However, all these methods will have to compete with EBV immortalisation and cell fusion which in turn are constantly improving, particularly as they start to incorporate ideas and techniques involving DNA manipulations. There are certain immune responses which are unique to humans, or which need to be characterised in humans, including antibodies involved in auto-immune diseases, and antibodies which maintain an active immune state in healthy individuals with certain parasitic, cancer or infectious diseases. Here the rescue by gene technology of human hybridomas or cell lines immortalised by EBV

virus, may provide a powerful way of defining the properties of such antibodies and immortalising them[68]. Like Ehrlich 90 years ago[84] ".....we have already caught a distinct glimpse of the land which we hope, nay, which we expect, will yield rich treasures for biology and therapeutics". We see a jungle of technologies, old and new, fertilising each other: in the immediate future, most of them start with immunised animals.

Acknowledgements We would like to thank A. Lesk and S. Pledger for help in preparing Fig. 1. and our colleagues for their advice.

Figures

Figure 1. Antibody structure.

The antibody (IgG) consists of four chains, two heavy and two light, which in turn are built by stringing together domains of similar architecture. Each chain is paired with another chain by lateral packing of the domains and also by at least one disulphide bond. Each domain consists of two β-sheets which pack together to form a sandwich, with exposed loops at the ends of the strands. Thus the C-domains have three β-strands in one sheet (strands C, F, G) and four strands (strands A, B, D, E) in the other. The V-domains have an extra two strands in one sheet (C, C', C", F, G). This framework is highly conserved in different antibodies. The three loops at the top of the V-domain are hypervariable in sequence and fashion the antigen-binding site[37]. Despite the sequence hypervariability, most of the antigen binding loops have a small repertoire of main chain conformations.

The antibody can be proteolytically cleaved at the flexible hinge region, yielding Fab fragments which bind antigen (comprising heavy chain VH and CH1 domains, top of the hinge and the entire light chain), and Fc fragments which bind to effector functions (comprising lower hinge and heavy chain CH2 and CH3 domains). The model is taken from the solved X-ray crystallographic structures of Fab and Fc domains of myeloma protein KOL[85], represented according to Lesk[86] and redrawn by an artist. Each strand of the β-sheets has been colour coded (see key to strand topology). The β-sheet defined by the A, B, D, E strands is more intensely coloured. The hypervariable regions (VH:1-3; VL:4-6) are in red; the binding sites for high affinity receptor[33], C1q[34] and carbohydrate attachment site are also marked in red, as triangles, circles or squares respectively.

Figure 2. Engineered antibodies and fragments.

A range of engineered antibodies and fragments are depicted. Each box represents a domain. Single chain Fv fragments (scFv) - in which VH and VL domains are linked by a peptide (see text) - and Fab fragments have been used to target enzymes and toxins. In immunoadhesins, a ligand specific for receptor (here CD4) is attached to an Fc fragment. Single VH domains (dAbs)[23] and even single CDRs (minimal recognition units or mru)[87,88] have been identified

with antigen binding activities. Site directed mutagenesis of antibodies and fragments has also been used to alter effector functions and improve affinities[41] (see text).

Figure 3 Organisation of V-genes

The domain structure of the antibodies is mirrored at the level of the gene, as the individual domains are encoded by separate exons. In turn the VH and VL domains are built from separate genetic elements, and assembled by DNA rearrangements during lymphocyte differentiation. Thus the first and second hypervariable loops are encoded by the germ-line V-genes, but the third hypervariable loop by the combination of V, D and J elements (for the VH domain) of V and J elements (for the VL domain). The third hypervariable loop of VH is accordingly the most diverse in sequence and backbone conformation and is often the longest of the loops. Exon structure is illustrated for the unrearranged and rearranged VH genes and is similar (not shown) for VL genes (Vκ or Vλ). VH (unrearranged or rearranged heavy chain variable region as appropriate), D (D-segment), J (J-segment), CH1, CH2, CH3 (first, second and third heavy chain constant exons respectively), h (hinge exon), L (signal sequence), p (Ig promoter = octanucleotide motif).

The potential diversity of the mouse primary repertoire is huge as a consequence of the combinatorial arrangements of the genetic elements. It can be estimated as follows. (1). Diversity due to combinatorial integration: 300 Vκ x 4 Jκ = 1.2 x 10^3 and 200 VH x 15 D x 4J = 12 x 10^3 (2). Diversity due to junctional alternatives (alternative readings at junction due to differences in reading frame, and lengths of J and D segments): Vκ to Jκ ~ 3 and VH to D to J ~ 40 (3). Diversity due to N-segment for heavy chain >10 (4). Hence combined diversity for each chain: light chains = 3 x 10^3 and heavy chains > 5 x 10^6 *Conclusion: diversity due to combinatorial association of heavy and light chains: > 10^{10}. Additional factor due to somatic mutation > 10^{30} (see text).*

Figure 4. PCR cloning of rearranged VH and VL genes into expression vectors.

Primer mixtures can be designed for PCR amplification of most families of V-genes, as the nucleotide sequences at the 5' and 3' ends of the rearranged V-

genes are relatively conserved[21]. At the 5' end, mixed PCR primers have been based within the signal sequence[65,67] or at the N-terminal end of the mature variable domain[21,66]. At the 3' end, PCR primers have been located within the J-region or the constant region. One set of PCR primers, located entirely within the rearranged V-gene exon, amplifies both mRNA and chromosomal DNA[21,23]. The PCR primers incorporate restriction sites and after amplification and digestion with restriction enzymes can be cloned into specialised plasmid or lambda phage vectors directly for expression of the V-genes. The cloning of VH and VL genes into a plasmid expression vector is illustrated here (L, leader sequence for secretion into bacterial periplasm and/or beyond outer membrane; R, polylinker cloning site; X and Y, extra polypeptide sequence (for example heavy chain CH1 domain, light chain CL domain or peptide tag for recognition by monoclonal antibody).

Figure 5. Strategies for cloning paired VH and VL genes from lymphocytes of an immunised animal.

VH and VL genes (expressed as pairs VHx/Vly) from n lymphocytes can be amplified by PCR from the mRNA or genomic DNA of single cells or populations. The lymphocytes can also be selected by antigen-binding, or immortalised as hybridomas by fusion with myeloma cells or by infection with EBV. From single lymphocytes, or clones, the original VHa/VLa gene combination is readily rescued. From populations of lymphocytes, repertoires of VH VL genes (VLn) could be combined at random, and antigen-binding combinations selected. However for large populations of lymphocytes, original combinations of VH and VL genes will be a minor proportion of heterologous combinations, some of which may display residual antigen binding activity. The library will contain $(n-1)^2$ VHxVLy elements, (n-1) each of VHxVLa and VHaVLy elements and a single VHaVLa element. VHa and VLa however may occur in multiple cells. At earlier stages of immunisation, there are likely to be some artificial pairs which originate from different clones, but which are able to bind antigen - particularly with responses dominated by "unique" VH and VL gene combinations (restricted or idiotypic responses[59,60]). After completion of this manuscript we received a preprint [90] which is in line with this prediction. As maturation for high affinity proceeds, the number of mutations increase and the likelihood of effective heterologous complementation diminishes.

Figure 6. Mimicking the immune system

VH and VL gene repertoires derived by a range of methods, such as reproduction of the available library from lymphoid cells, assembly of V, D and J elements, or gene cross-over (to mimic gene conversion). The genes are cloned and expressed, and VH/VL pairs binding to antigen can be selected or screened, for example on membrane filters. Here the VH/VL pair, binding to antigen (Ag) is tagged with a C-terminal peptide which is recognised by a monoclonal antibody (mAb), or is expressed on the surface of a bacterium or phage. For detection, the mAb or Ag can for example, be coupled to radioisotope, or to an enzyme for production of a coloured dye. (A) capture of VH/VL with antigen and detection with mAb. Other schemes could involve capture by binding to membrane and detection with Ag or capture by mAb and detection with Ag. (B) capture with Ag and detection of phage plaques. The genes encoding VH/VL pairs, identified as antigen-binding, can be rescued and hypermutated to form the secondary antibody library. These genes can then be expressed and screened for improved binding to antigen. This process will need to be repeated several times to acheive the efficiency of animal immune systems.

Devising selection systems for catalytic antibodies may be a different story. Such antibodies have been made through hybridoma technology by immunisation with transition state analogues, or by chance[2,3]. However, animals select antibodies on the basis of binding, not catalysis, and the transfer of V-gene repertoires to bacteria gives a new twist to the field[24]. Vast libraries might be screened directly, not for binding, but for catalysis for example with substrates yielding fluorescent products, or via complementation of bacterial mutants deficient in a catalytic step of interest. In this case antibodies will need to be expressed intracellularly[89].

Table 1. Immortalisation of antibodies using unselected cells from hyperimmunised animals

	Hybridomas	EBV transformation	Random combinatorial gene cloning (bacteria)
Species restrictions	Yes	Only human	No
Chain pairs	H + L	H + L	H and L separate
Positive/negative Clones	1/50	1/500 (?)	$<1/10^4$ ("Artificial")* $<1/10^8$ ("Original")
Assay on plates (clones as replicas or overlays per plate)	~300	N.D.	$\sim 10^{3-5}$ (?)
Assays on supernatants	well established, very sensitive	well established, less sensitive	possible
Doubling time of cells	18 hr	~40 hr	30 min
Stability	Good	Poor	Good
Product	Full molecules	Full molecules	Fragments
Yield of product	20 - 100 µg/ml	0.5 - 5 µg/ml	0.5 - 10 µg/ml

* These values are calculated as follows. If P is probability of finding a particular combination of light (L) and heavy (H) chains in an N-size combinatorial library, and P_L and P_H are the probabilities of random occurrence of each component, $P = 1 - e^{-q}$ where q is NP_LP_H. If we assume that 1% of the mRNA molecules encode antigen-specific Ig ($P_L = P_H = 10^{-2}$), there is ~ 60% probability that $1/10^4$ recombinants will contain one H and one L chain encoding any antigen-specific Ig: the vaste majority are comprised of artificial combinations, which may or may not specify antigen-binding activity. If there is a dominant lymphocyte clone which represents 1% of the antigen-specific Ig lymphocytes, the frequency of each chain is 10^{-4} and testing 10^8 clones will give us a 60% probability of finding the "original" combination. However regardless of whether they originate from antigen-binding lymphocytes, L chains may be capable of complementing the H chains from antigen-specific Ig. This frequency may be high particularly in primary responses and early secondary responses, before extensive diversification through hypermutation, but will depend on the cut-off affinity used in the assay . Taking a value of 1:100 (ref. 69), there is a 60% chance that ~ 10^{-4} of the recombinants will give an "artificial" positive signal.

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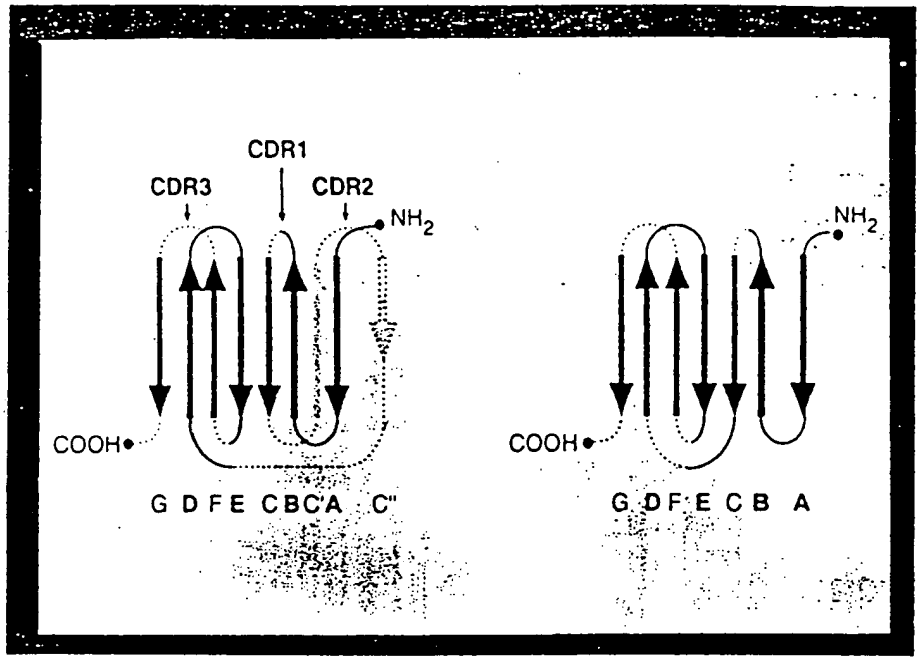
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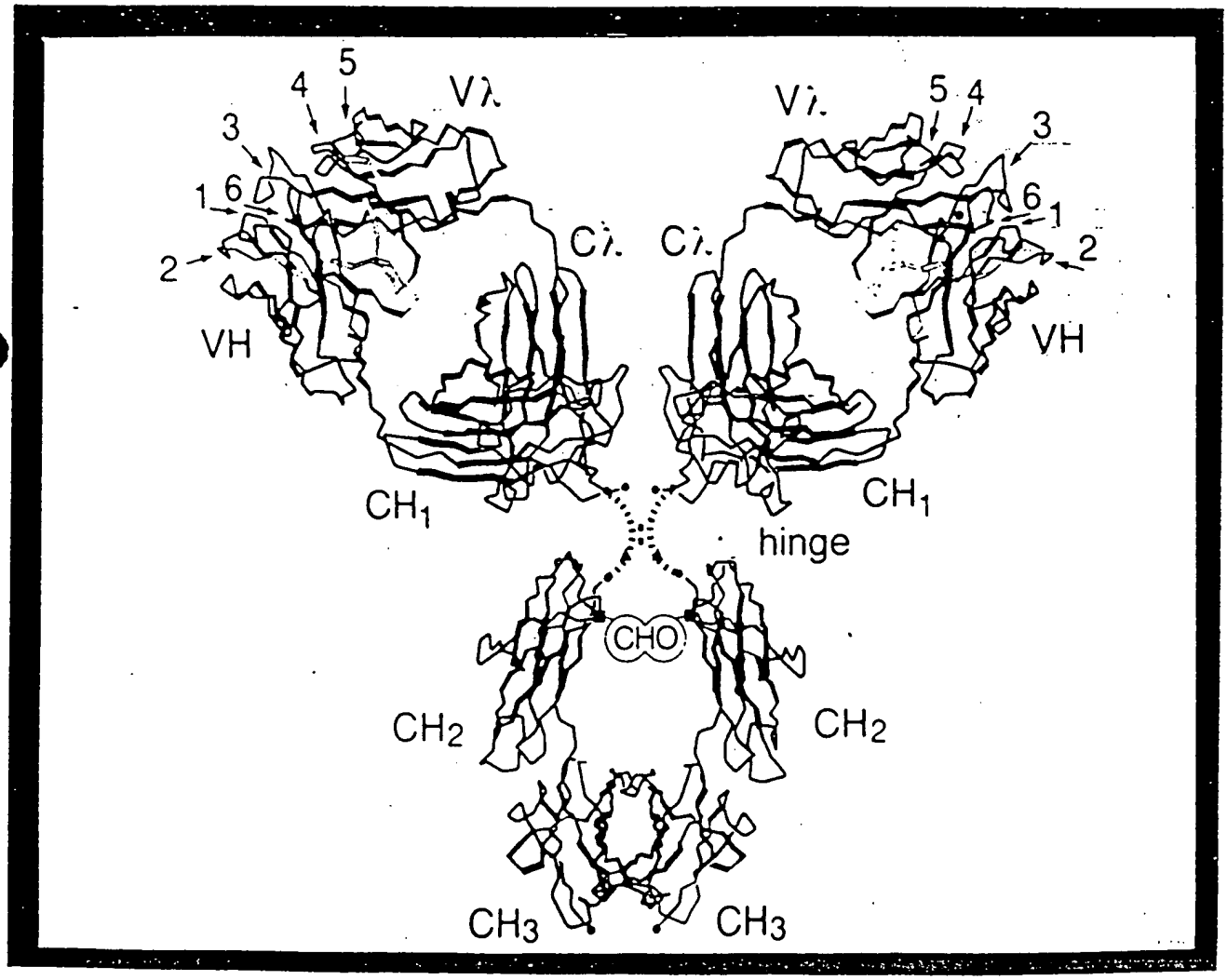
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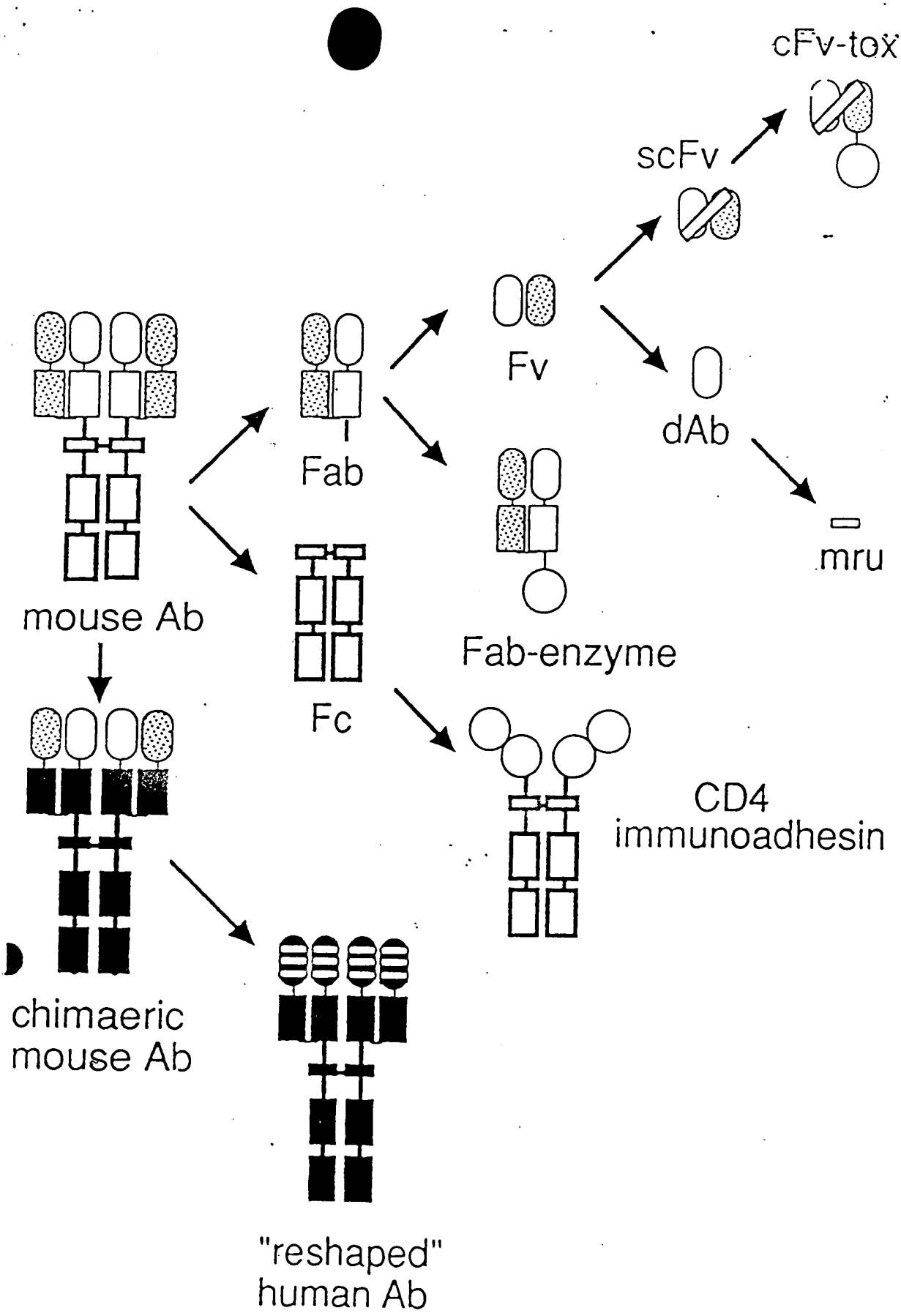
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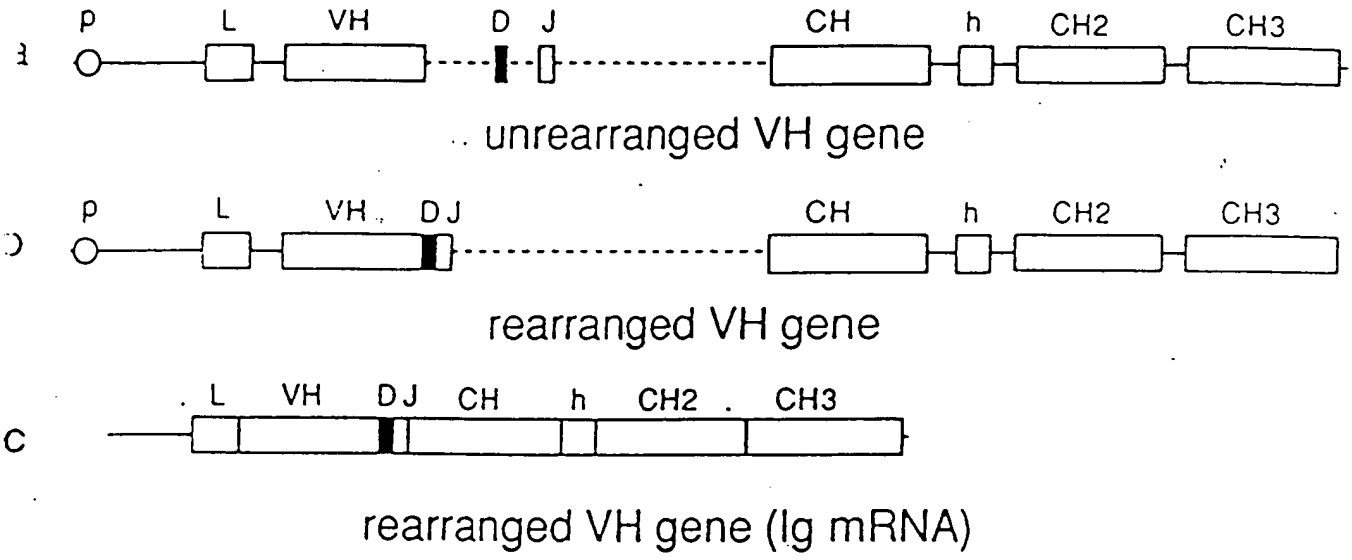
Fig 2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rearranged VH gene (Ig mRNA)

Fig 3

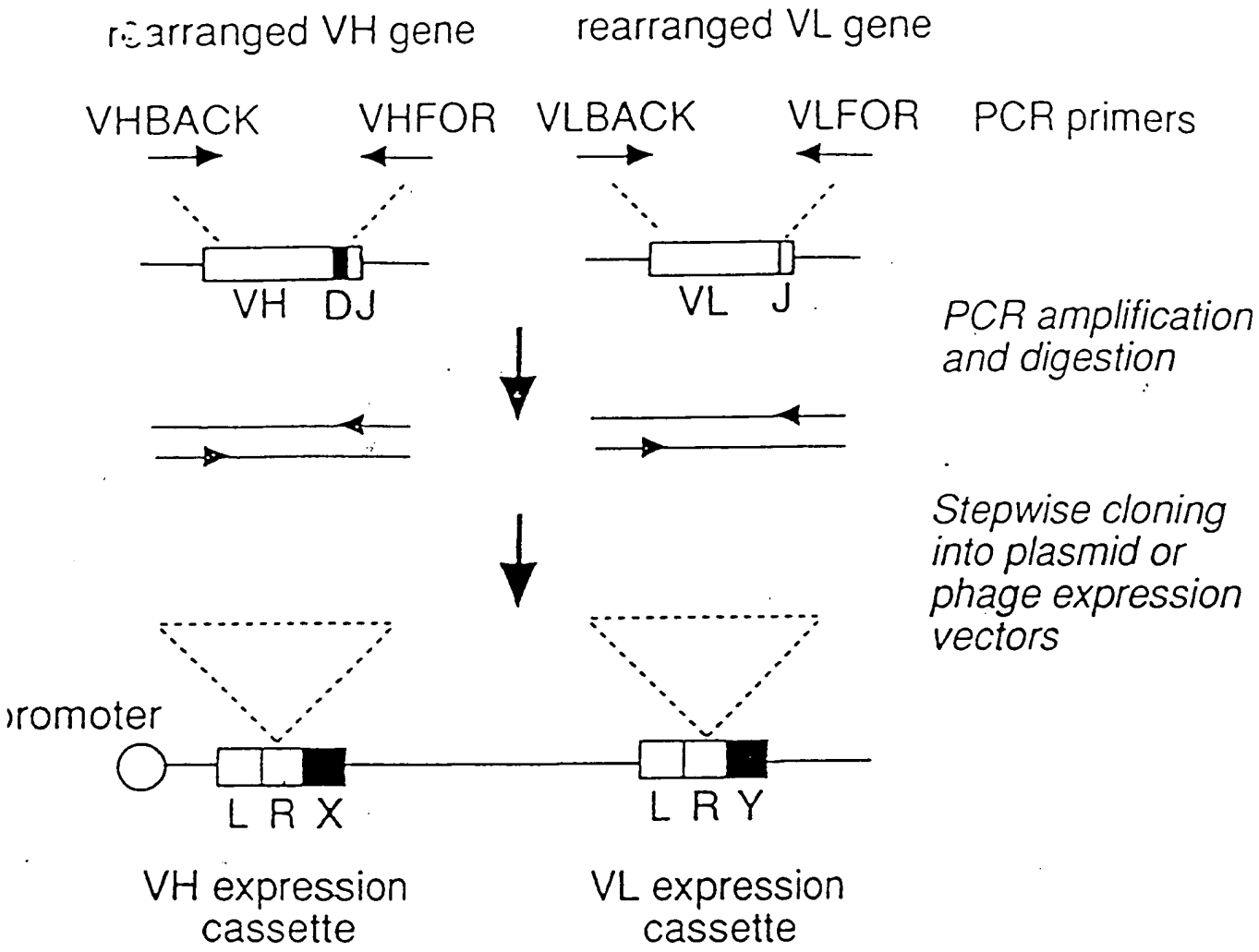
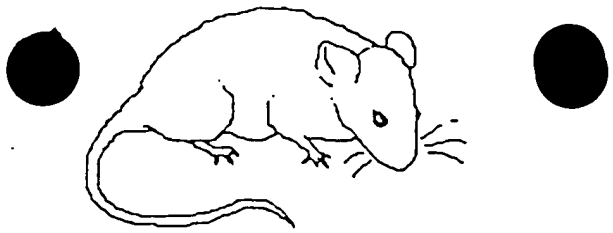

rearranged VH gene
rearranged VL gene
VHBACK
VHFOR
VLBACK
VLFOR
PCR primers
VH DJ
VL J
PCR amplification and digestion
Stepwise cloning into plasmid or phage expression vectors
romoter
L R X
L R Y
VH expression cassette
VL expression cassette

FIG 4



Harvest lymphocytes from immunised animal

LYMPHOCYTES OR HYBRIDOMAS

Selection of antibody-producing cells

POPULATIONS OF CELLS

Total (unselected) or small (antigen-selected) combinatorial libraries

SINGLE CELLS OR CLONES

PCR amplification of VH and VL genes

VHx VLy

VHa VLa

Assembly of VH and VL pairs

LIBRARY WITH ELEMENTS

VHxVLy, VHxVLa, VHaVLy, VHaVLa

ORIGINAL COMBINATION

VHaVLa

Screen for antigen-binding activities

VHa? /VLa?

VHa/VLa

Fig 5

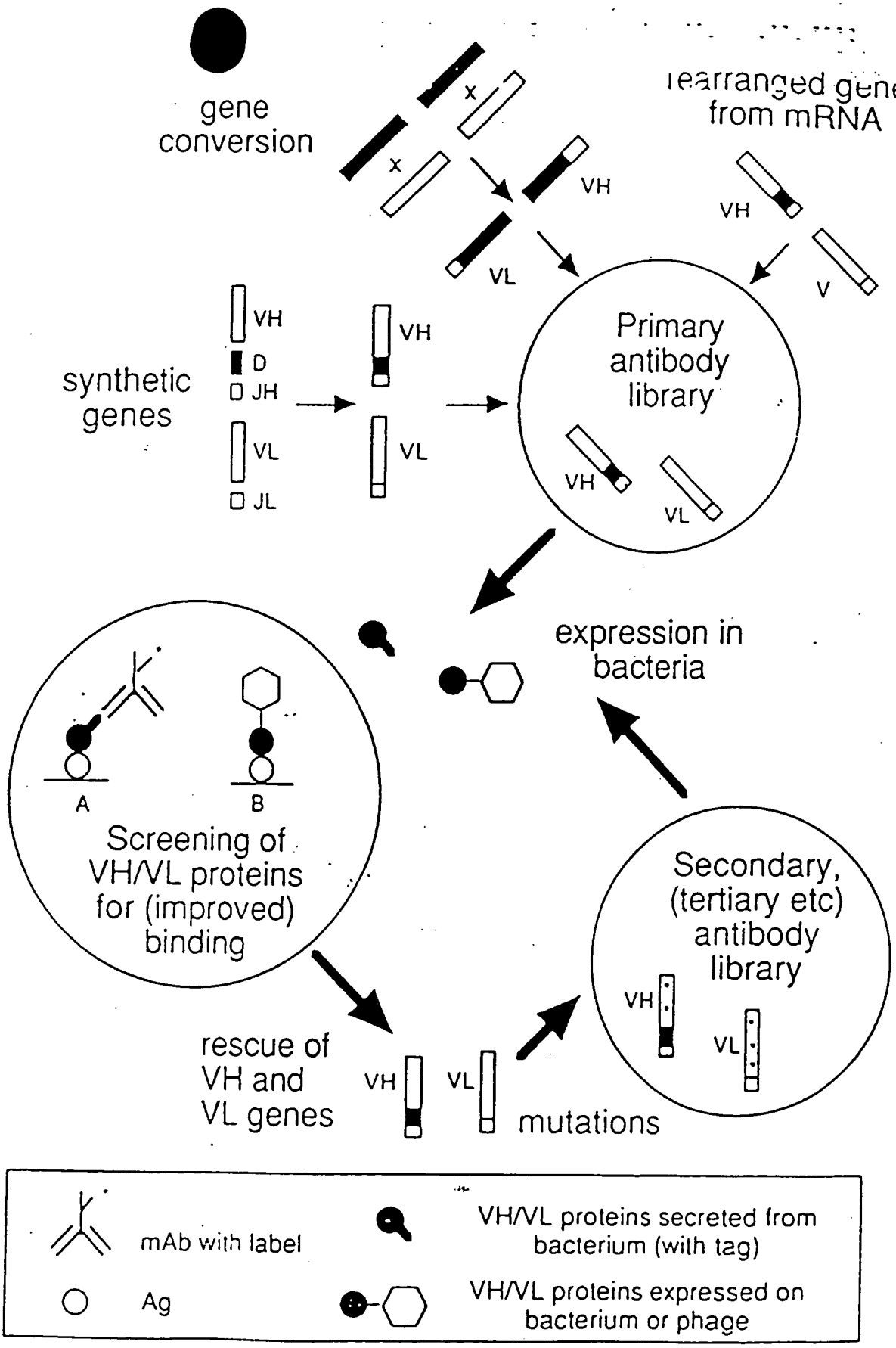

gene conversion
x
x
VH
VL
rearranged genes from mRNA
VH
V
synthetic genes
VH
D
JH
VL
JL
VH
VL
Primary antibody library
VH
VL
expression in bacteria
A
B
Screening of VH/VL proteins for (improved) binding
Secondary, (tertiary etc) antibody library
VH
VL
rescue of VH and VL genes
VH
VL
mutations
mAb with label
Ag
VH/VL proteins secreted from bacterium (with tag)
VH/VL proteins expressed on bacterium or phage

Fig 6